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Platelet Antistaphylococcal Responses Occur through $P2X_1$ and $P2Y_{12}$ Receptor-Induced Activation and Kinocidin Release▽

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Platelets (PLTs) act in antimicrobial host defense by releasing PLT microbicidal proteins (PMPs) or PLT kinocidins (PKs). Receptors mediating staphylocidal efficacy and PMP or PK release versus isogenic PMP-susceptible (ISP479C) and -resistant (ISP479R) *Staphylococcus aureus* strains were examined in vitro. Isolated PLTs were incubated with ISP479C or ISP479R (PLT/*S. aureus* ratio range, 1:1 to 10,000:1) in the presence or absence of a panel of PLT inhibitors, including P2X and P2Y receptor antagonists of increasingly narrow specificity, and PLT adhesion receptors (CD41, CD42b, and CD62P). PLT-to-*S. aureus* exposure ratios of ≥10:1 yielded significant reductions in the viability of both strains. Results from reversed-phase high-performance liquid chromatography indicated that staphylocidal PLT releasates contained PMPs and PKs. At ratios below 10:1, the PLT antistaphylococcal efficacy relative to the intrinsic *S. aureus* PMP-susceptible or -resistant phenotype diminished. Apyrase (an agent of ADP degradation), suramin (a general P2 receptor antagonist), pyridoxal 5′-phosphonucleotide derivative (a specific $P2X_1$ antagonist), and cangrelor (a specific $P2Y_{12}$ antagonist) mitigated the PLT staphylocidal response against both strains, correlating with reduced levels of PMP and PK release. Specific inhibition occurred in the presence and absence of homologous plasma. The antagonism of the thromboxane A_2, cyclooxygenase-1/cyclooxygenase-2, or phospholipase C pathway or the hindrance of surface adhesion receptors failed to impede PLT anti-*S. aureus* responses. These results suggest a multifactorial PLT anti-*S. aureus* response mechanism involving (i) a PLT-to-*S. aureus* ratio sufficient for activation; (ii) the ensuing degranulation of PMPs, PKs, ADP, and/or ATP; (iii) the activation of $P2X_1$/$P2Y_{12}$ receptors on adjacent PLTs; and (iv) the recursive amplification of PMP and PK release from these PLTs.

Mammalian platelets have unambiguous characteristics of antimicrobial host defense effector cells (37, 38). Among their antimicrobial armamentarium, these cells release platelet microbicidal proteins (PMPs) that directly kill microbial pathogens and mediate phagocyte chemotaxis. We previously discovered that human PMPs include the CXC chemokines platelet factor 4 and platelet basic peptide and derivatives, such as connective tissue-activating peptide 3 (CTAP-3) and interleukin-8, as well as the CC chemokine RANTES (*r*eleased upon *a*ctivation, *n*ormal *T*-cell *e*xpressed and *s*ecreted) (34, 37, 43). Microbicidal chemokines from platelets have been termed platelet kinocidins (PKs) (45), reflecting dual and complementary host defense roles likely bridging innate and adaptive immunity.

Mature platelets possess distinct granule types that contain molecules conferring the hemostatic and host defense roles of these cells (39). Their dense granules (δ-granules) store mediators of vascular tone, including serotonin, ADP, and precursors of eicosanoids and thromboxanes, as well as calcium and phosphate. Lysosomal (λ) granules store enzymes that principally mediate thrombus dissolution. Along with proteins involved in modulating coagulation and endothelial cell repair, platelet α-granules also contain the array of known PMPs and PKs in rabbit and human platelets, which appear to be integral to antimicrobial host defense (34, 37).

Platelets possess a diverse array of constitutive and inducible membrane receptors that are highly sensitive and rapidly responsive to a broad spectrum of agonists associated with tissue injury or infection. For example, platelets interact with bacteria directly and indirectly through a variety of receptor-ligand interactions (37). Human platelets are rapidly bound and aggregated in vitro by organisms that commonly gain access to the bloodstream, including *Staphylococcus aureus* (41). In turn, platelets liberate their granular contents, including the PMPs and PKs and nonpeptide agonists that may stimulate responses in adjacent platelets. Yet, the mechanisms that evoke platelet antimicrobial responses are unclear. As *S. aureus* is among the most predominant endovascular pathogens, the consequences of its interactions with platelets likely play a significant role in shaping infection or immunity. Therefore, the aim of the present studies was to identify a receptor-mediated pathway(s)

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TABLE 1. Summary of antagonists and inhibition of platelet antistaphylococcal response[a]

Antagonist	Concn[c]	Mechanism of action	Inhibition of platelet[b] staphylocidal effect on:	
			ISP479C	ISP479R
APY	5 U/ml	Extracellular ADP hydrolase	+	+
SUR	100 μM	P2X and P2Y ADP receptor antagonist	+	+
PND	300 μM	$P2X_1$ receptor-specific antagonist	+	+
CNG	100 nM	$P2Y_{12}$ receptor-specific inhibitor	+	+
IND	25 μM	COX-1 and COX-2 antagonist	−	−
YOH	10 μM	β-Adrenergic receptor antagonist	−	−
SQX	25 μM	TXA_2 receptor antagonist	−	−
PRO	200 μM	Phospholipase C pathway antagonist	−	−
PAP	100 μM	$P2Y_1$ receptor-specific antagonist	−	−
Anti-CD41 (abciximab)	2.5 μg/ml	MAb directed against GPIIb/IIIa (CD41)	−	−
Anti-CD42b	2.5 μg/ml	MAb directed against GPIb (CD42b)	−	−
Anti-CD62P	2.5 μg/ml	MAb directed against P-selectin (CD62P)	−	−

[a] Antagonists are divided into effective and ineffective groups. Quantitative analyses of antagonists found to inhibit platelet staphylocidal responses are summarized in Fig. 2, 3, and 4. Abbreviations and symbols: SQX, SQ29548; MAb, monoclonal antibody; +, inhibition; −, no inhibition.
[b] Log_{10} platelet/*S. aureus* ratio of 3 (1,000:1) (Fig. 1).
[c] Where appropriate, study concentrations were chosen for relevance to human therapeutic levels.

through which *S. aureus* elicits platelet antimicrobial responses involving the liberation of PMPs and PKs.

MATERIALS AND METHODS

Organisms. A well-characterized isogenic pair of *S. aureus* organisms, ISP479C (PMP-susceptible parent) and ISP479R (PMP-resistant derivative), was studied. Strain ISP479R is a stable mutant generated from parental strain ISP479C by transposon mutagenesis as detailed previously (11). The PMP-resistant phenotype derives from the disruption of the *snoD* gene, encoding a complex I NADH oxidoreductase (2). These strains have differential susceptibility phenotypes when exposed to thrombin-induced PMP-1 (tPMP-1) in vitro (35). ISP479R exhibits reduced killing by low levels of tPMP-1 in vitro (e.g., ≥90% survival of a 10^3 CFU/ml inoculum following 2 h of exposure to 2 μg of tPMP-1 at 37°C), compared with that of tPMP-1-susceptible ISP479C (≤25% survival under identical test conditions). These strains have been described in detail previously (24, 26, 36).

Organism preparation. *S. aureus* strains ISP479C and ISP479R were cultured in brain heart infusion broth (Difco Laboratories, Detroit, MI) and incubated for 3 h at 37°C under aerobic conditions to achieve logarithmic-phase growth. Logarithmic-phase organisms were harvested by centrifugation, washed in phosphate-buffered saline (pH 7.2), briefly sonicated to ensure singlet cells, quantified by spectrophotometry (600 nm; validated by quantitative culture), and suspended in minimal essential medium (MEM) without glutamine (pH 7.2; Irvine Scientific, Santa Ana, CA) to the desired concentration (see below).

Platelet preparation. Platelets were collected and isolated by standard methods as we have described previously (24, 42). In brief, fresh whole blood was obtained by venipuncture of New Zealand White rabbits and collected into polypropylene tubes containing sodium citrate as an anticoagulant (1:5, vol/vol). Rabbit platelets were studied, as they are the most fully characterized platelets in terms of their antistaphylococcal roles and interactions with *S. aureus* (3, 11, 24, 26, 33, 36, 37, 46). Centrifugation (100 × *g*) produced an upper platelet-rich plasma suspension; the upper two-thirds of this fraction yielded a platelet-rich suspension having <1% leukocyte contamination. Platelet-rich plasma was transferred into polypropylene tubes and centrifuged (250 × *g*), yielding a loose platelet pellet. For experiments, isolated platelets were washed in MEM buffer, quantified by spectrophotometry (600 nm) as validated by hemacytometry, and suspended in MEM to the desired study concentration (see below). Routine monitoring by the assessment of aggregation, morphology, and P-selectin expression was used as a control to ensure that no significant activation of platelets occurred during preparation.

Influence of platelet-*S. aureus* ratio on staphylocidal response. One objective of this study was to assess the stoichiometry of platelet-*S. aureus* exposure as related to the extent of the platelet staphylocidal response. To do so, platelets and the *S. aureus* ISP479C or ISP479R strain were mixed in MEM across a range of ratios from 10,000:1 (log_{10} 4) to 1:1,000 (log_{10} −3), and the mixtures were incubated at 37°C for 30 min. Following incubation, polyanetholesulfonate (0.1%; Sigma Chemicals) was added to cease potential further PMP- or PK-mediated killing, as described previously (36, 42). Next, *S. aureus* survival was determined by the quantitative culture of sonicated aliquots on blood agar and the enumeration of CFU after incubation for 24 h at 37°C. In parallel, samples were centrifuged and supernatants were analyzed for the PMP or PK content as described previously (see below).

Platelet antagonism. Platelet antagonists with established mechanisms of action ranging from general to highly specific were used to probe putative pathways involved in the platelet antistaphylococcal response. The panel of study antagonists and their targets of action are summarized in Table 1. With the exception of cangrelor (CNG; kindly provided as a gift from The Medicines Company [Parsippany, NJ]), antagonists were commercially available and used per manufacturer instructions. Likewise, monoclonal antibodies directed against platelet CD41 (GPIIb/IIIa [abciximab; Lily]), platelet CD42b (GPIb; Abcam), and platelet CD62P (P-selectin; Abcam) surface receptors were prepared and used as directed by the suppliers. Platelet antagonism studies were conducted at a standard platelet-to-*S. aureus* ratio of 1,000:1 (demonstrated to reproducibly yield ≥95% staphylocidal efficacy [see Fig. 1]). Where indicated, platelets (10^8) were exposed to a given antagonist for 30 min at 37°C in MEM, washed, and resuspended in fresh MEM prewarmed to 37°C. In repeated pilot studies, no significant differences were observed using this method versus allowing the antagonist to remain during the interaction period. Selected antagonists were also compared as described above in the presence versus the absence of 5, 10, 50, and 100% (vol/vol) homologous plasma. Controls for potential direct anti-*S. aureus* activity of the antagonists were included in all experiments. For antagonism studies, *S. aureus* ISP479C or ISP479R in MEM was then added to achieve a platelet-*S. aureus* exposure ratio of 1,000:1 (10^8 platelets:10^5 bacteria). For experiments involving apyrase (APY), this antagonist was added immediately upon the mixing of platelets and *S. aureus*, as it inhibits extracellular ADP. Bacterium-platelet incubations were for 30 min at 37°C and were terminated by polyanetholesulfonate as described above, and aliquots were quantitatively cultured on blood agar. CFU were enumerated as before, and the results for antagonist-exposed versus control platelets were compared for staphylocidal efficacy against *S. aureus* ISP479C and ISP479R.

Platelet secretion of microbicidal proteins or kinocidins. The release of PMPs or PKs from platelets exposed to *S. aureus* in the presence or absence of the platelet antagonists was studied. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on selected supernatants obtained following platelet-bacterium interactions versus respective control platelets. Methods of RP-HPLC detection of PMPs or PKs are detailed elsewhere (3, 34, 42, 46). Analyses were performed with paired supernatants from independent experiments, and the relative PMP or PK quantities and composition profiles were semiquantitatively compared by peak area.

Statistical analyses. All experiments were performed a minimum of three times independently with at least two replicates each time, on different days and with different platelet donor sources. A two-way analysis of variance was used to compare differences in anti-*S. aureus* platelet efficacy or the effects of platelet antagonists. The Bonferroni correction for multiple comparisons was used where appropriate. *P* values of ≤0.05 were considered statistically significant.

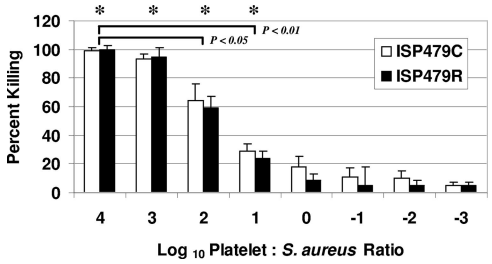

FIG. 1. Influence of platelet ratio on antistaphylococcal efficacy. Logarithmic values of platelet-to-staphylococcus ratios ranged from 4 (10,000:1) to −3 (1:1,000). The limit of assay detection was considered to be 5% ± 2.5% killing (41). Compared with the viability of controls not exposed to platelets, significant reductions in the viability of exposed bacteria occurred at platelet-to-*S. aureus* ratios of ≥10:1 (∗, $P \leq 0.05$). Platelet-to-bacterium ratios of 10,000:1 and 1,000:1 were not significantly different in antistaphylococcal efficacy. As indicated, the levels of killing of the initial *S. aureus* inoculum were significantly different for platelet-to-*S. aureus* ratios of $\log_{10} 4$ and $\log_{10} 2$ ($P < 0.05$) or $\log_{10} 4$ and $\log_{10} 1$ ($P < 0.01$).

RESULTS

Influence of platelet/*S. aureus* ratio on staphylocidal response. A ratio-response effect on the staphylocidal capacity of platelets was observed (Fig. 1). Platelet-to-*S. aureus* ratios of ≥1,000:1 ($\log_{10} 3$) yielded extensive (>95% killing), equivalent staphylocidal effects on ISP479R and ISP479C strains. At 100:1, platelets caused approximately 60% killing of the initial inoculum of either strain. At a ratio of 10:1, platelets caused 30 and 24% killing of ISP479C and ISP479R, respectively. Thus, the platelet-to-*S. aureus* ratio appears to have an impact on the staphylocidal efficacy (e.g., ratio of 10,000:1 or 1,000:1 versus 100:1, $P < 0.01$; ratio of 10,000:1 or 1,000:1 versus 10:1, $P < 0.05$) (Fig. 1). A trend toward greater reduction in the viability of strain ISP479C than in that of strain ISP479R was seen at platelet-to-*S. aureus* ratios of 100:1 or lower; these differences did not achieve statistical significance. At ratios of ≤1:1, platelets exerted no significant staphylocidal efficacy under the conditions studied (Fig. 1).

Influence of platelet antagonism on staphylocidal response. Platelet antagonists differed in their effects on the platelet staphylocidal response (Table 1). The antagonists APY, suramin (SUR), pyridoxal 5′-phosphonucleotide derivative (PND), and CNG significantly interfered with platelet staphylocidal responses. Of these, APY mitigated the response, while SUR, PND, and CNG essentially abolished the response (Fig. 2) (P, <0.05 versus the response of control platelets). The inhibitory effect of these antagonists occurred regardless of whether *S. aureus* strain ISP479C or ISP479R was used as the challenge organism. Moreover, $P2X_1$ and $P2Y_{12}$ receptor-specific inhibition of the platelet antistaphylococcal response was demonstrated in the presence of up to 50% homologous plasma for ISP479C and up to 10% plasma for ISP479R (Fig. 3). In contrast, the antagonists yohimbine (YOH), propanolol (PRO), indomethacin (IND), SQ29548, and 3′-phosphoadenosine-5′-phosphosulfate (PAP) failed to alter the platelet staphylocidal responses against either study strain in the presence or absence of plasma, compared with the responses of untreated platelets (data not shown). Likewise, none of the platelet adhesion receptor antagonists (CD41 [GPIIb/III], CD42b [GPIb], or P-selectin [CD62P]) reduced the staphylocidal efficacy of platelet-*S. aureus* interactions (Fig. 4).

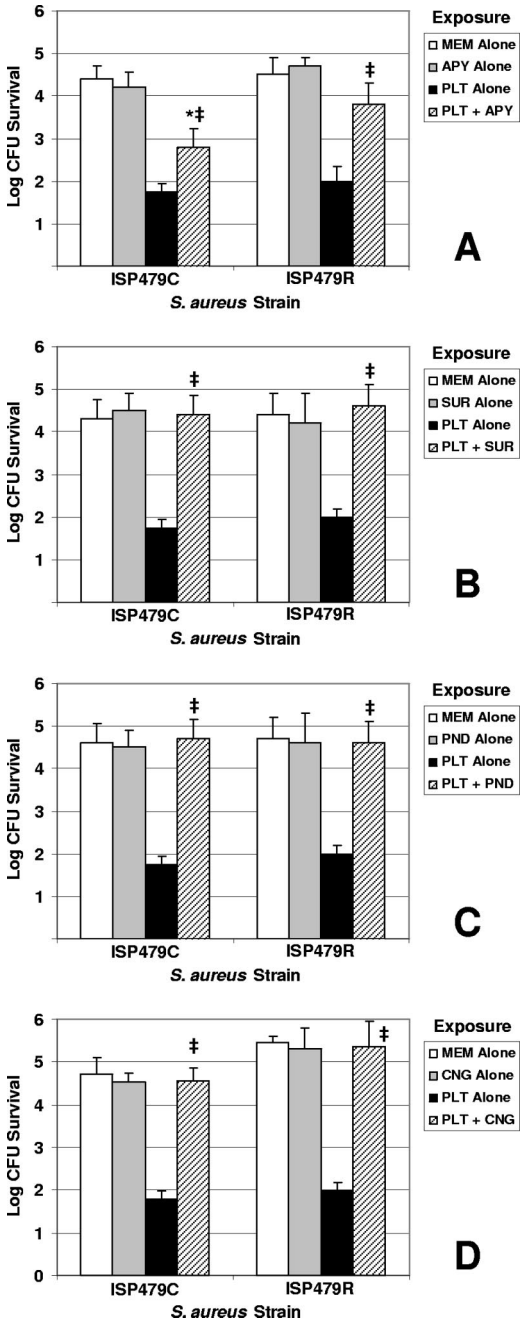

FIG. 2. Inhibition of platelet antistaphylococcal efficacy. As detailed in Materials and Methods, platelets (PLT) were preexposed to antagonists, washed, and mixed with *S. aureus* bacteria at a ratio of 1,000:1 (10^8 platelets:10^5 bacteria; found to yield >95% staphylocidal efficacy). Geometric means of data from a minimum of three independent experiments are shown. ∗, P of <0.05 versus platelets alone; ‡, P of <0.05 versus antagonist-exposed platelets.

PMP and PK release versus platelet staphylocidal efficacy. Platelet staphylocidal responses against either *S. aureus* strain were associated with the release of polypeptides identified by RP-HPLC retention times as those characteristic of known

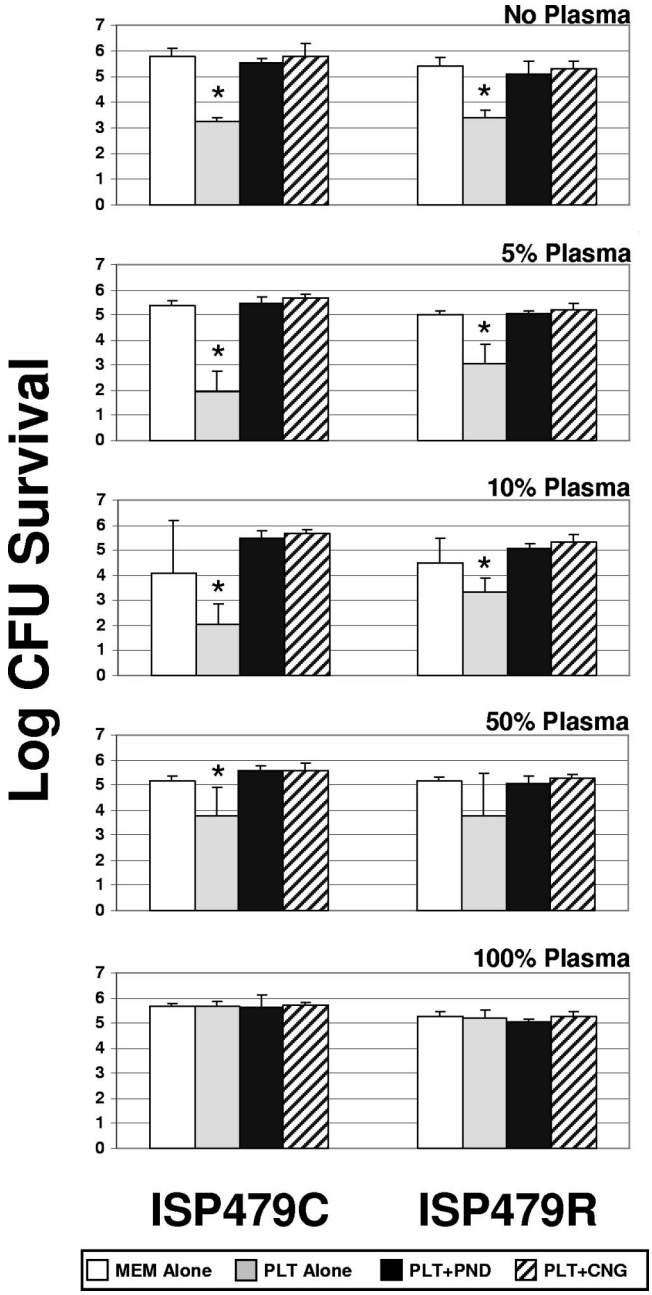

FIG. 3. Influence of plasma on platelet anti-*S. aureus* response antagonism. As detailed in Materials and Methods, platelets (PLT) were mixed with *S. aureus* bacteria at a ratio of 1,000:1. Geometric mean values reflect data from a minimum of three independent experiments. Consistent with other data presented herein (e.g., Fig. 2), PND and CNG inhibited the platelet antistaphylococcal response in the presence of plasma. *, P of <0.05 versus platelets alone.

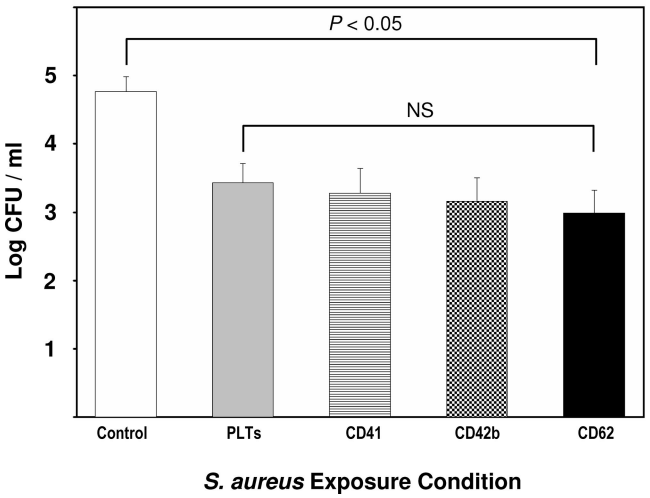

FIG. 4. Effects of platelet surface adhesin antagonists on antistaphylococcal responses. Platelets pretreated with anti-surface receptors were mixed with *S. aureus* bacteria at a ratio of 1,000:1 (platelet-to-bacterium ratio, $\log_{10}$ 3). Geometric means from a minimum of three independent experiments using the PMP-susceptible strain ISP479C are shown. Consistent with other data presented in this study, exposure to platelets alone achieved a significant reduction in CFU (a decrease of log 1.43 ± 0.28 CFU equates to 96.7% ± 9.5% killing; *, P of <0.05 compared with the results for the control inoculum). In contrast, none of the adhesin receptor antagonists investigated significantly interfered with the antistaphylococcal responses compared with the response of platelets alone. NS, not statistically significantly different.

PMPs and PKs (Fig. 5A). Importantly, the inhibition of PMP or PK release corresponded with the inhibition of staphylocidal efficacy. For example, the $P2X_1$ inhibitor PND prevented the liberation of antistaphylococcal PMPs and PKs, paralleling the absence of the staphylocidal efficacy of platelets exposed to this inhibitor (Fig. 5C). In comparison, the $P2Y_1$ inhibitor PAP failed to prevent PMP or PK release and did not impede the platelet staphylocidal response (Fig. 5B). The relative abundance of prototypic PMPs and PKs in reaction supernatants corresponded to the staphylocidal efficacies of the platelet-to-*S. aureus* ratios tested (data not shown). Thus, PMPs and PKs were more abundant in supernatants from platelet-*S. aureus* exposure ratios of 100:1 or greater than in those from ratios of lower than 10:1 (Fig. 1). The PMP and PK profiles evoked from platelets following exposure to *S. aureus* ISP479C or ISP479R did not differ significantly, and the significant liberation of these molecules from unstimulated platelets was not observed.

DISCUSSION

Platelets have multifunctional roles now believed to contribute significantly to antimicrobial host defense. These include (i) navigation to and accumulation at sites of tissue infection or injury; (ii) direct and opsonic interaction with microbial pathogens, including viruses, bacteria, fungi, and protozoa; (iii) the generation of reactive oxygen species such as superoxide, hydroxyl, and peroxide; (iv) the degranulation and release of PMPs and PKs; and (v) the potentiation of leukocyte antimicrobial functions (see references 23, 37, and 38 for reviews).

The classical studies of Clawson and coworkers (6–9) showed that interactions with bacteria evoke a series of platelet events proceeding from shape change to aggregation. Along this progression, platelets transform from uniform disks into amoeboid cells with pseudopodia, coincident with microtubule organization and degranulation. At the molecular level, platelets have multiple surface receptors that detect and modulate

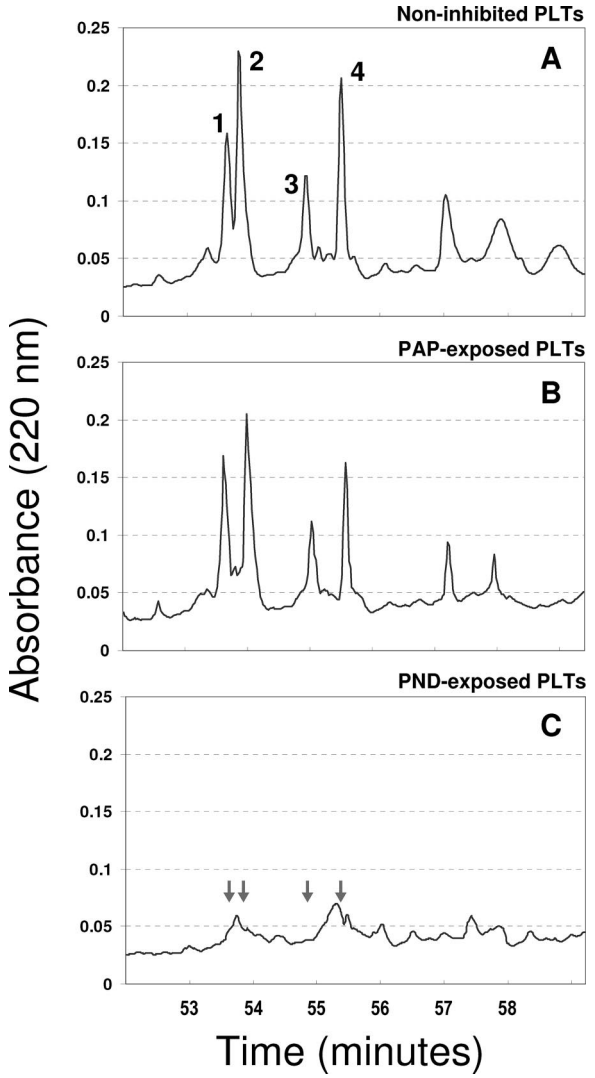

FIG. 5. Release of PMPs in response to *S. aureus*. Comparative RP-HPLC chromatograms are shown for platelet (PLT) supernatants following exposure to *S. aureus* alone (A), PAP ($P2Y_1$ ADP receptor antagonist) and *S. aureus* (B), or PND (P2X receptor-specific antagonist) and *S. aureus* (C). The predominant constituents liberated from platelets (PLTs) in response to *S. aureus* ISP479C (log platelet-to-organism ratio, 3) correspond to *N*-serine and *N*-aspartate versions of PMP-1 (A, peaks 1 and 2). Peaks 3 and 4 are consistent with platelet basic peptide and its derivative, connective tissue-activating peptide 3 (CTAP-3). This profile is characteristic of previously documented PMPs or PKs (37, 38, 42, 46). Arrows in panel C indicate the expected major PMP/PK elution peaks in response to *S. aureus*, compared with those in the absence of platelet inhibitors (A).

responses to diverse stimuli. Endogenous signals transduced by these receptors include adenosine nucleotides (ADP and ATP), thromboxanes (e.g., thromboxane A_2 [TXA_2]), epinephrine, platelet-activating factor, and prostaglandins (see reviews in references 37 and 38). Numerous recent studies have revealed ligands through which *S. aureus* interacts with platelets, including integrin IIb/IIIa fibrinogen receptor (3); clumping factor A (32); thrombospondin (18); fibrin (27); and staphylococcal protein A, immunoglobulin G, and platelet Fc receptors (16). Our prior data also found that staphylococci bind directly to washed human or rabbit platelets in vitro and that the platelet-to-organism ratio affects the velocity and extent of *S. aureus*-induced platelet aggregation (33, 41). Thus, the objective of the present study was to examine the mechanism of platelet response, rather than *S. aureus* determinants that may initiate this response.

The present studies indicate that the platelet-to-*S. aureus* interaction ratio is an important variable in the platelet antistaphylococcal response. Normal platelet counts in humans range from 150,000 to 400,000 per microliter of blood; this concentration translates to 1.5×10^8 to 4.0×10^8 platelets/ml (28). By comparison, typical *S. aureus* bacteremia levels in patients rarely exceed 10^2 to 10^3 organisms/ml (10). Based on these facts, platelet-to-*S. aureus* interaction ratios on the order of 10^5:1 or lower would be physiologically relevant in *S. aureus* bloodstream challenge. Important in this respect, the present studies demonstrated ≥95% antistaphylococcal efficacy at ratios of 10,000:1 (10^4:1) or 1,000:1 (10^3:1), approximately 60% killing at 100:1 (10^2:1), and approximately 25% efficacy at a ratio of 10:1. These data are congruent with the effects of profound thrombocytopenia on antimicrobial host defense in vivo. For example, Sullam et al. (33) observed that animals rendered selectively thrombocytopenic (but not neutropenic) have significantly worse infective endocarditis due to a PMP-susceptible strain of viridans group streptococci than animals with normal platelet counts. Human studies have also shown thrombocytopenia to be an independent risk factor for infection in organ transplant patients and other patient populations (5, 12, 35). To our knowledge, no prospective studies have specifically addressed potential relationships between platelet antagonism and an increased risk of infection due to *S. aureus*. These relationships are presently under investigation in our laboratories.

The present finding that APY reduced platelet staphylocidal efficacy implicated P2 purine and pyrimidine receptors as contributing to the anti-*S. aureus* responses of platelets. To define the specific P2 receptor(s) contributing to this response, established antagonists targeting specific platelet receptors were evaluated for their potential to interfere with platelet staphylocidal efficacy. There are two general types of P2 receptors on platelets: (i) P2X, ligand-gate/ion channel P2 receptors (ionotropic), and (ii) P2Y, G protein-linked P2 receptors (metabotropic) (reviewed in reference 4). The principal P2 subtypes include P2X subtype 1 ($P2X_1$), P2Y subtype 1 ($P2Y_1$), and P2Y subtype 12 ($P2Y_{12}$; also known as P2Y[AC] or P2Y[PLC]) (4, 19, 20, 31). Via the respective P2X and P2Y receptors, ATP and ADP likely play at least two important roles in platelet activation (19): (i) induce the expression of conditional receptors (e.g., IIb/IIIa) and (ii) mediate a cascade activation sequence in which ATP or ADP release activates bystander platelets. Mechanistically, in platelets ATP and ADP both cause increases in free intracellular calcium, originating from internally sequestered stores and the influx of extracellular calcium via calcium channels (14, 15, 21). Changes in calcium concentration prompt microtubule assembly and platelet shape change, as well as granule organization and degranulation. These events are likely required for PMP and PK liberation and staphylocidal efficacy.

The observations that APY and the broad P2X/P2Y inhibitor SUR inhibited platelet staphylocidal efficacy prompted us

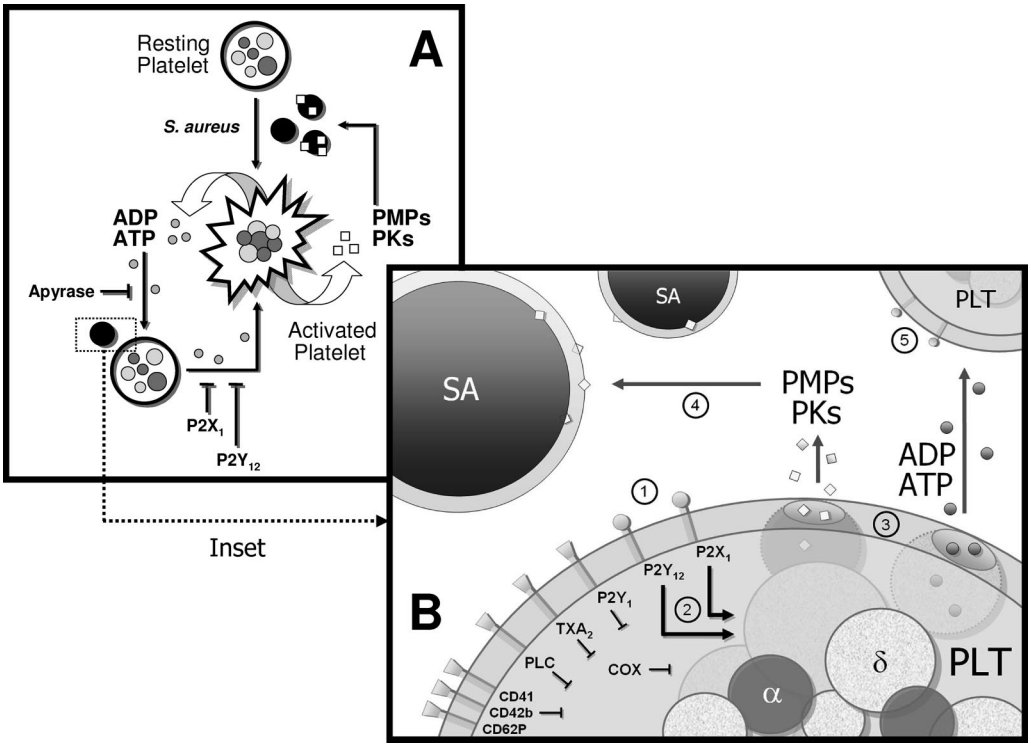

FIG. 6. Model of platelet antistaphylococcal response mechanisms. As supported by current data, the model illustrates how platelets may be activated to respond in parallel pathways that promote staphylocidal efficacy. (A) At the cellular level, the interaction with *S. aureus* evokes distinct responses in resting platelets: the liberation (and putative processing) of PMPs and PKs, which exert direct microbicidal effects on the organism, and the secretion of adenosine nucleotides (ADP/ATP), triggering a recursive cascade for the activation of adjacent platelets. Note that inhibitors of the ADP/ATP platelet activation pathway preclude the platelet staphylocidal response. (B) Detailed aspects of the model at the molecular level are illustrated. The degradation of extracellular ADP by APY or the inhibition of P2X or $P2Y_{12}$ adenosine nucleotide receptors by SUR (a general P2 inhibitor), PND (a high-affinity $P2X_1$ inhibitor), or CNG (a high-affinity $P2Y_{12}$ inhibitor) specifically prevents platelet (PLT) staphylocidal efficacy. In contrast, the antagonism of $P2Y_1$, phospholipase C (PLC), TXA_2, or COX pathways or the CD41, CD42b, or CD62P platelet adhesin receptor did not impede the staphylocidal responses of platelets. Thus, the antistaphylococcal efficacy of platelets involves a self-amplifying and recursive sense/response mechanism: (1) direct or indirect interactions of platelets and *S. aureus* (SA); (2) platelet activation, with autocrine or intercrine $P2X_1$ or $P2Y_{12}$ receptor-mediated signal transduction prompting granule mobilization; (3) the degranulation and liberation of ADP/ATP from δ-granules; (4) the deployment of direct antimicrobial effector molecules (PMPs and PKs) from α-granules; and (5) the adenosine nucleotide-mediated activation of adjacent platelets, with the ensuing amplification of antimicrobial responses. The observed pattern of relationships between platelet-to-*S. aureus* exposure ratios and staphylocidal efficacy is suggestive of a threshold platelet ratio necessary to sustain an intercrine platelet cascade required to achieve PMP/PK concentrations sufficient for staphylocidal efficacy.

to use increasingly specific P2X and P2Y receptor antagonists to probe P2 receptors that may participate in the platelet antistaphylococcal responses (Fig. 2). Platelets exposed to PND failed to exert a staphylocidal response to either *S. aureus* strain. These results were consistent with $P2X_1$ receptor function in the response but did not necessarily exclude $P2Y_1$- and/or $P2Y_{12}$-mediated pathways. Normally, stimulation by G protein-coupled P2Y receptors evokes latent phospholipase C pathways, yielding inositol-2,3,5-phosphate, diacylglycerol, and ensuing calcium mobilization from internal stores (17). Thus, two approaches were used to specify P2Y receptor subclasses involved in the response to *S. aureus*. The ADP analogue PAP was used, as it is among the most well characterized $P2Y_1$ receptor-specific antagonists. Platelets treated with PAP had normal staphylocidal efficacy. However, platelets exposed to the specific high-affinity $P2Y_{12}$ antagonist CNG exhibited no staphylocidal efficacy. These findings indicate that the $P2Y_{12}$ but not the $P2Y_1$ receptor preferentially contributes to platelet antistaphylococcal responses. Consistent with these observations, Quinton et al. recently demonstrated that the $P2Y_{12}$ receptor plays a dominant role compared with that of $P2Y_1$ in agonist-induced platelet α-degranulation (29). Moreover, specific $P2X_1$ and $P2Y_{12}$ receptor inhibition of platelet antistaphylococcal efficacy was demonstrable ex vivo in up to 50% homologous plasma (Fig. 3), reflecting the percentage of plasma found in normal human whole blood (30). Collectively, these findings indicate a relevant role for ADP/ATP-driven $P2X_1$/$P2Y_{12}$ receptor amplification of PMP/PK release in vivo and suggest that this mechanism is favored at sites of platelet intensification sufficient for the cascade stimulation of adjacent platelets (e.g., thrombi) rather than systemic circulation (26).

The present studies also used specific approaches to define possible pathways downstream of receptor activation that may be involved in the platelet antistaphylococcal response. By inhibiting cyclooxygenase-1 (COX-1) and COX-2, IND prevents the conversion of arachidonic acid to prostaglandins (24). In contrast, TXA_2 is a platelet agonist derived from phospholipid oxidation and arachidonic acid metabolism (13). Neither

IND nor the specific inhibitor of the TXA_2 receptor, SQ2954, altered the staphylocidal responses of platelets (data not shown). Platelet α-adrenergic and phospholipase C pathways can also generate secondary messengers important to platelet responses (29). However, neither YOH nor PRO interfered with the platelet staphylocidal response compared to that of controls (data not shown). Collectively, these results indicate that COX, TXA_2, α-adrenergic, and phospholipase C pathways are not integral to platelet staphylocidal responses. Likewise, platelet surface adhesion receptors GPIb (CD42b), GPIIb/IIIa (CD41), and P-selectin (CD62P) do not appear to be involved in the platelet staphylocidal response mechanism (Fig. 4). This pattern of data suggests that platelet antistaphylococcal responses are not directly correlated to the adhesion of organisms to platelets by known receptor-ligand interactions (3, 37, 38).

As a correlate of efficacy, platelet supernatants were analyzed for known PMPs and PKs (34, 36, 40, 44–46). Our prior in vitro studies demonstrated that platelets release these peptides in response to thrombin or *S. aureus* exposure under physiological conditions (1, 34, 42). The present investigations identified peptides characteristic of known PMPs and PKs in supernatants with staphylocidal activity (Fig. 5). Platelet $P2X_1$ and $P2Y_{12}$ receptor inhibitors (but not $P2Y_1$ inhibition) substantially reduced the quantities of these PMPs and PKs in *S. aureus*-induced platelet releasates, corresponding to reduced antistaphylococcal efficacy. These observations are consistent with PMP and PK release and processing in platelet staphylocidal functions (43, 46). However, no substantial differences in the profile of PMPs or PKs liberated from platelets in response to *S. aureus* strains ISP479C and ISP479R were detected. This observation suggests that the net staphylocidal efficacy of the platelet response is related primarily to the intrinsic PMP—or PK—susceptibility of the challenge organism, rather than an organism-specific propensity to evoke the release of these molecules. These interpretations are consistent with the data in previous reports on the concentrations of PMPs or PKs required for staphylocidal effects (34, 36, 39). Given that platelets may interiorize *S. aureus* or like pathogens during their antimicrobial response (47), it is conceivable that such organisms are exposed to high concentrations of these peptides within platelet engulfment vacuoles.

In summary, the present results provide new information regarding mechanisms by which platelets exert antistaphylococcal responses. The findings indicate that platelet staphylocidal responses involve feedback amplification resulting from ATP stimulation of $P2X_1$ receptors and ADP stimulation of $G\alpha_i$-coupled signal transduction pathways mediated through $P2Y_{12}$ receptors (4, 22, 25) (Fig. 6). In turn, such stimulation appears to prompt successive platelet activation and degranulation, resulting in the liberation of PMPs and PKs. Investigations of the specific *S. aureus* factor(s) involved in either promoting or impeding platelet antistaphylococcal host defenses are ongoing in our laboratory.

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